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ORIGINAL ARTICLE

Effects of eluted components from 4-META/MMA-TBB adhesive resin sealer on osteoblastic cell proliferation

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Abstract *Background/purpose:* Adhesive resin sealer systems are applied to seal root-canal systems more effectively through the formation of a resin impregnation layer. The purpose of this study was to investigate the effects of eluted components of the adhesive, Super-Bond sealer (SBS), on the proliferation of osteoblastic cells *in vitro*.
Materials and methods: The standard powder:liquid ratio according to the manufacturer's instructions was used to produce a cylindrical block of SBS (5 mm in diameter, 10 mm long) for this elution study. The resin block was placed on a 100-mm culture dish. Osteoblastic cells were seeded at a density of 4×10^6 in α-minimum essential medium containing 10% fetal bovine serum and cultured in a humidified incubator. After 3 days of culture with or without SBS, cells were retrieved and lysed according to the manufacturer's instructions. The cellular events induced by the eluted components from SBS were analyzed using an antibody assay for mitogen-activated protein kinases (MAPKs) and a 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Furthermore, the concentration of boron, a component of the catalyst, tri-*n*-butyl borane (TBB), was analyzed using inductively coupled plasma optical emission spectrometry.
Results: Expression of MAPKs increased after SBS application. The MTT assay indicated that TBB, one of the components of SBS, accelerated the proliferation of osteoblastic cells. Values of boron were 1.66 ± 0.37 and 1.74 ± 0.30 ppm in cells cultured with and without FBS, respectively.

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Conclusions: The eluted components from SBS can increase the expression of some MAPKs related to osteoblastic cell proliferation and differentiation *in vitro*. Both the elution experiment and treatment of cell culture with SBS components indicated that the boron originating from TBB is likely to be responsible for activation of the proliferation of osteoblastic cells.

Introduction

The purpose of root-canal treatment is to prevent and/or treat pulp and periapical pathoses.[1] Irrigation and medication usually yield sufficient infection control of root-canal systems. Secondary infection is generally prevented by the final sealing of the root canal with various types of filling materials. Therefore, root-canal filling to seal the root-canal system from the outside environment is the most important step in ensuring a good long-term prognosis.

The traditional root-canal filling material is composed of a gutta-percha core. However, gutta-percha characteristically demonstrates a high coefficient of thermal expansion and shrinkage. It also does not adhere to or seal root dentine. This means that a sealer cement consisting of zinc oxide and eugenol, calcium hydroxide, or epoxy resin is required to provide resistance to bacterial invasion after root-canal filling.[2–5]

Resin systems that adhere to dentine are also applied for endodontics. These adhesion technologies were originally borrowed from restorative dentistry. A 4-methacryloxyethyl trimellitate anhydride/methacrylate- tri-*n*-butyl borane (4-META/MMA-TBB) adhesive resin (Super-Bond; SB) developed in Japan (Sun Medical, Shiga, Japan) is widely used in dentistry as a bonding agent.[6] Adhesive resins are used to seal dentine through the creation of a resin-impregnated layer[7] that protects the pulp from the actions of oral fluids and their contaminants. Recently, an SB sealer (SBS) containing radiopaque zirconium oxide was developed as a root-canal sealer.[8] This modified type of SB adheres to root canal dentine and is supposed to seal the root-canal system more effectively by forming a resin-impregnated layer.[7]

Several studies have examined the cytotoxicity and tissue responses after SB application *in vitro* and *in vivo*.[9–13] Its effect on the expression of phosphoinositol-3-kinase was recently detected using a complementary (c)DNA microarray and reverse-transcription polymerase chain reaction (RT-PCR).[14] However, little is known about how SBS affects mitogen-activated protein kinases (MAPKs) related to cell proliferation and differentiation, and which components of SBS directly influence osteoblastic cell growth. In the present study, the effects of SBS were examined using an antibody assay for the phosphorylation of MAPKs, and a 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze cell proliferation in osteoblastic cells cultured with each component of SBS. An analysis of boron released from SBS *in vitro* was also carried out to investigate the effects of boron on cellular activity.

Materials and methods

Antibody assay for the phosphorylation of MAPKs

The standard powder-liquid ratio (0.13 g sealer powder, 0.08 g monomer [4 drops], and 0.006 g catalyst [1 drop]) was used according to the manufacturer's instructions to produce cylindrical blocks of SBS (5 mm in diameter and 10 mm long). The resin block was placed on a 100-mm culture dish. Osteoblastic (NOS-1)[15] cells derived from a human osteosarcoma were seeded at a density of 4×10^6 in α-minimum essential medium containing 10% fetal bovine serum (FBS), and were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and air. After 3 days of culture with or without SBS, cells were retrieved and lysed according to the manufacturer's instructions.

Relative levels of phosphorylation of various signal transduction kinases were analyzed using a human phospho-MAPK assay kit (R&D Systems, Minneapolis, MN, USA) that examines 21 kinds of MAPK.[16] A phospho-site-specific biotinylated antibody was used to detect 20 kinds of phosphorylated kinases via streptavidin-horseradish peroxidase, the membranes were exposed to x-ray film, and detected signals were developed on the film. Thereafter, each signal was quantitatively measured using the densitometric mode of a ChemiImager (Alpha Innotec, Altishofen, Switzerland).

MTT assay

NOS-1 cells were seeded at a density of 10^4 cells in 96-well flat-bottomed plates at 100 μL/well. The culture was stimulated with varying concentrations of 4-META (0.125—10 mM), MMA (0.125—10 mM), and TBB (0.125—10 mM) dissolved in culture medium supplemented, or not, with FBS. Unstimulated cells were used as controls. Cell proliferation in response to different treatments was detected using an MTT Cell Growth Assay Kit (Millipore, Billercia, MA, USA). After incubation for 3 days (at 37°C with 5% CO_2), 10 μL of MTT reagent was added to the wells, and cells were incubated for an additional 4 h at 37°C. After incubation, the resulting formazan crystals were dissolved by adding an MTT solubilizing solution. Within 1 h, the absorbance at 570/630 nm was recorded using an enzyme-linked immunosorbent assay plate reader (Model550, Bio-Rad, Tokyo, Japan). Data from control samples were considered to be 100%, and those obtained for cells treated with various concentrations were converted to percentages compared to the control. Differences between the control and

TBB-stimulated cells were compared using Student's *t* test. A P value of <0.05 was considered statistically significant.

Analysis of boron concentrations

A method similar to the above-mentioned methods for the MAPK assay was used to analyze boron concentrations in culture medium containing components released from cylindrical blocks of SBS. The boron concentration was analyzed using an inductively coupled plasma optical emission spectrometer (limit of detection, <10 ppb) (ICP SPS7700; Seiko Instruments, Chiba, Japan). Standard boron levels were obtained by means of a standard curve constructed using 0, 1, 5, 10, and 20 ppm boron. All measurements were performed in duplicate.

Results

Fig. 1 shows the density of the reaction products using a human phospho-MAPK array. Five of the 21 MAPKs examined exhibited a difference in their expression ratio between the experimental and control groups of ≥ 1.2. Ratios of extracellular signal-regulated kinase (ERK)1, ERK2, and Akt1/2/pan were 3.0, 1.2, and 1.6/1.9/1.2, respectively (from duplicate samples).

MTT assays showed that supplementation with 4-META or MMA in the culture medium with and without FBS did not affect cell proliferation at any of the concentrations examined (Fig. 2A,B). There was a more-prominent inhibitory tendency for cells cultured with FBS than for those cultured without FBS. This tendency was also confirmed in the TBB group (Fig. 2C). The highest (percentage) of proliferation was found for stimulation with 0.125 mM TBB ($P < 0.01$). Values at concentrations of <0.125 mM were lower than that at 0.125 mM TBB (data not shown).

The amount of boron was calculated from the medium sample cultured with SBS. Values were 1.66 ± 0.37 and 1.74 ± 0.30 ppm in cells cultured with and without FBS, respectively.

Discussion

This study was carried out to demonstrate the effects of the components dissolved from SBS on osteoblastic cells in culture. Phosphoinositide 3-kinases (PI3Ks), which represent a family of lipid kinases, are key mediators of intracellular signaling in many cell types.[17] PI3Ks are upstream regulators of a number of signaling cascades that control cell proliferation, growth, death, migration, metabolism, and a host of other biological responses.[18] Furthermore, the PI3K/Akt pathway is one of the most critical signaling pathways involved in regulating cell survival.[19,20] For example, the tumor growth factor-β2-induced epithelial-mesenchymal transition in postoperative remnants of lens epithelial cells is mediated by downregulation of connexin 43, which is regulated through the PI3K/Akt pathway.[21] Data from a real-time PCR[14] and the present phosphorylation antibody assay of MAPKs indicate that the PI3K/Akt/ERK pathway is activated by components from the SBS.

Our laboratory previously reported that the application of SBS to alveolar bone in rats using a canal model resulted in a mild postoperative inflammatory reaction, and that new bone formation occurred in the periapical space near the SBS on Day 5 (unpublished data). Thereafter, osteoblastic cells were used for an MTT assay to examine the effect of SBS. In an MTT assay using human pulp cells to

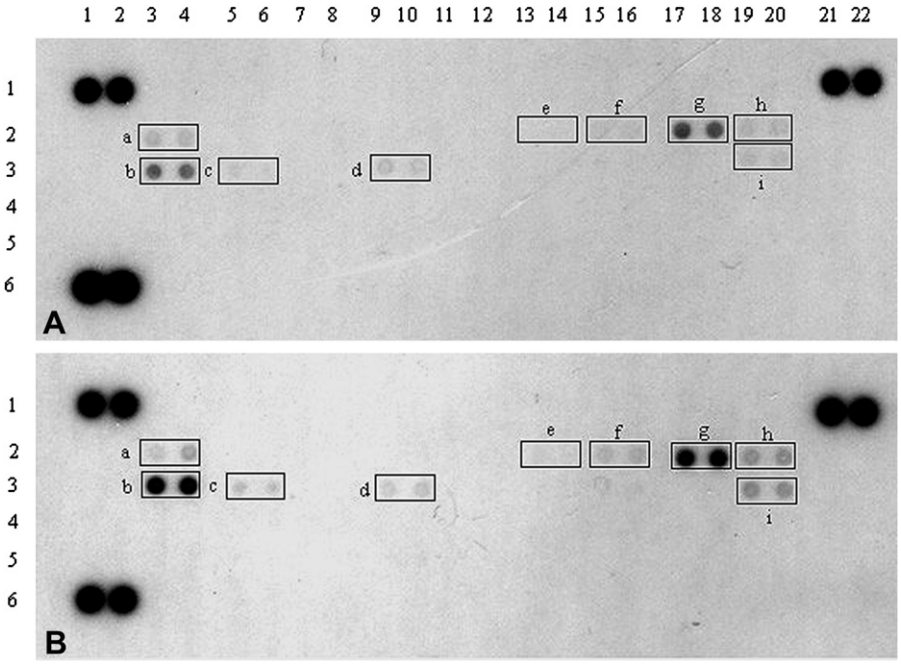

Figure 1 Signals detected on a membrane spotted with various antibodies against phosphorylated MAPKs following incubation with cell lysates from untreated cultures (A) or SBS-treated cultures (B). The signals indicated by the squares are as follows; a: ERK1, b: ERK2, c: JNK2, d: p38 α, e: ribosomal S6 kinase (RSK)1, f: glycogen synthase kinase (GSK)-3α/β, g: Akt 1, h: Akt 2, i: Akt pan.

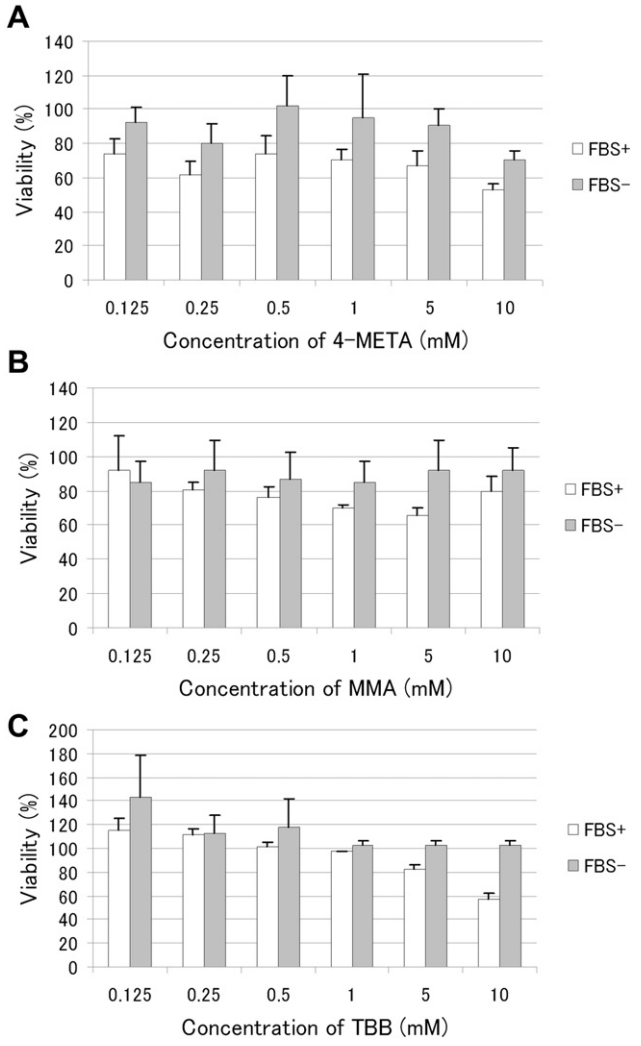

Figure 2 The viability of NOS-1 cells was determined by MTT assay following incubation with (A) 4-META, (B) MMA, and (C) TBB. The data represent the mean ± standard error of values taken from three wells.

examine effects of SB on cell viability, eluates from SB were found to be the least cytotoxic agents compared to other resin cements.[22] The cytotoxicity of 4-META/MMA-TBB was comparable to that of MMA-TBB. However, TBB induced a higher cytotoxicity than 4-META/MMA.[12] The cytotoxicity induced by the 4-META resin may primarily be associated with TBB.[12] The concentration of TBB (>1000 ppm) used in this study was extremely high, and is unlikely to be reached in clinical settings. In fact, our present data from the culture medium clearly demonstrate that the boron released from TBB was at a level of only a few ppm. Therefore, it is highly unlikely that a concentration of >1000 ppm of TBB release would ever occur under clinical conditions.

The present data show that cells cultured without FBS show higher MTT values than those cultured with FBS. This means that FBS may interact with components of the SBS, block and mask their effects. TBB-initiated polymerization has unique and different characteristics compared to those initiated by other common initiators: the decrease in residual MMA is rapid, is sustained for a long time, and results in a very low value of MMA.[23] This TBB effect was probably responsible for promoting cell proliferation.

Boron is a vital micronutrient in plants[24] and may be essential for animal growth and development. The role of boron in plants has been extensively investigated,[25] including its importance in pollination,[26] in the stability of the membrane,[27] and for increasing crop sizes.[28] However, little is known about boron homeostasis and function in animal cells.

NaBC1, the mammalian homolog of AtBor1, is a borate transporter. In the absence of borate, NaBC1 conducts Na^+ and OH^- (H^+), while in the presence of borate, NaBC1 functions as an electrogenic, voltage-regulated, Na^+-coupled $B(OH)_4^-$ transporter.[29] At low concentrations (0.1—0.5 mM), borate activates MAPK pathways to stimulate cell growth and proliferation in rat submandibular gland acini and ducts, although it is toxic at high concentrations (of >1 mM).[29] Furthermore, in a mineralization assay of MC3T3-E1 cells, increased mineralized nodules were observed in cells treated with boron (1 and 10 ng/ml) after 30 days of culture.[30] In the short term (24 h), boron decreased the cell-survival rate beginning at concentrations of ≥1 ppm. After long-term (72 h) culture, no statistically significant difference was detected in cells treated with different boron concentrations (2—10 ppm).[30] A relatively low concentration (1.6—1.7 ppm) of boron was demonstrated in the present study. Furthermore, the present analyzed values were closely consistent with the concentration (≈1.35 ppm) of boron showing the largest increases in cell proliferation observed in the MTT assay, as a concentration of 1 mM of TBB contains about 10.8 ppm boron. Thereafter, the boron released from TBB as a catalyst in SBS was confirmed to be potentially useful for accelerating osteoblastic cell proliferation.

In conclusion, the present study demonstrated that the eluted components from SBS can increase the expression of some MAPKs related to osteoblastic cell proliferation and differentiation *in vitro*. Both the elution experiment and the treatment of cells with each SBS component in culture that indicated that the boron originated from TBB may be responsible for activation of the proliferation of osteoblastic cells.

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